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REVIEW ARTICLE

Immunotoxicity of Metal Oxide and Metal Nanoparticles and Animal Models to Evaluate Immunotoxicity of Nanoparticles

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Abstract: Since metal (silver and gold) and metal oxide (TiO_2, ZnO, SiO_2) nanoparticles (NPs) are widely used in diagnostics, therapeutics, and commercial products, specialized immuno-toxicity studies are necessary to ensure their safe use in humans. In this article, we review the immunological toxicity of engineered nanomaterials (ENMs), including metal oxide and metal NPs, and suggest animal models of immunological disease suited to study the immunological effects of NPs. Administration of NPs at sub-toxic doses in animals with a background of immunological diseases, especially those with allergic or inflammatory diseases, will accelerate our understanding of the precise immunotoxicity and immune-stimulatory effects of NPs following real-life exposures.

Keywords: Nanoparticles, metal, metal oxide, immunotoxicity, animal model.

INTRODUCTION

Metal (silver and gold) and metal oxide (TiO_2, ZnO, SiO_2) nanoparticles (NPs) are widely used as nanodiagnostics and nanotherapeutics in the pharmaceutical field, and in-commercial products such as paint, coating, and cosmetics. Thus, various methods have been suggested for assessing their toxicity and immunomodulatory effects [1, 2], including the modified forms of Lusters' method [3]. However, assessment of toxicity for engineered nanomaterials (ENMs) is complicated by their unique physical characteristics such as their extremely small size and biocompatible surface modification. Further, the interaction between ENMs and the immune system, as well as the resulting pharmacokinetic and phenotypic responses, are critical factors in determining the balance between toxicity and clinical efficacy for many NPs [4].

Considering the widespread potential applications of ENMs in human health, specialized *in vitro* and *in vivo* immuno- and hemato-toxicity tests are necessary in addition to traditional toxicological assays. In particular, persistent inflammation plays a key role in the initiation of allergic diseases or inflammatory metabolic diseases. For instance, in bronchial asthma, persistent allergic inflammation induces airway remodeling that progresses to intractable asthma. Some NPs such as alum are reported to enhance T helper 2 cell (T_H2)-mediated immune responses via activation of eosinophil and induction of IgE [5], while crystalline SiO_2 NPs can induce IgG1 and IgE [6, 7]. Recently, the molecular and immunological mechanisms of action of NPs in allergic responses are receiving increasing attention. Although not all particles induce T_H2 immune responses, specific signals induced by NPs have been suggested. For example, inflammasome and damage-associated molecular patterns (DAMPs) evoked by NPs in immune cells may be involved in triggering T_H2 immune responses. The NOD-like receptor pyrin containing 3 (NLRP3) inflammasome promotes the production of IL-1β and IL-18, and is activated by SiO_2 NPs. DAMPs such as monosodium urate and host DNA are released from damaged cells and are recognized by intracellular DNA sensors that initiate inflammation by stimulating interferon gene expression [8, 9]. Further, prostaglandin E_2, released by macrophages in response to NPs, activates IgE induction by B cells [7]. Therefore, further research focusing on *in vivo* immuno-toxicological models is required to advance the understanding of the therapeutic and toxicological effects of NPs in biological systems.

In this article, we review the immunological toxicity of ENMs, specifically metal oxide and metal NPs, and suggest animal models of immunological diseases suited to identify the immunological effects of NPs *in vivo*. Administration of NPs at sub-toxic doses in these models will more precisely predict the immuno-modulatory effects of NPs in human patients.

IMMUNE CELLS AND METAL OXIDE OR METAL NPS

Humans are mostly exposed to NPs through the skin, respiratory tract, gastrointestinal tract, or by injection for medical purposes. Macrophages, dendritic cells, and mast cells comprise the first line of defense against NP exposure in the innate immune response. When NPs permeate into the circulation, they are phagocytosed by macrophages and den-

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dritic cells, which enable antigen presentation and stimulate adaptive immunity. NPs then interact with lymphocytes and can contribute to a broad spectrum of effects, including inflammation, hypersensitivity, and immunomodulation in adaptive immunity. NP core composition, size, concentration, and the duration of cell exposure are important factors for these immunological effects.

Macrophages

Our earlier study in THP-1 cells (human monocytic cell line) showed that silver NP (AgNP)-induced production of IL-8 (CXCL8), a typical chemokine, was dependent on particle size. 5 nm particles induced more cytotoxicity, IL-8 production, and reactive oxygen species (ROS) than 20 nm particles [10]. mRNA microarray analysis revealed IL-8 to be the prominent cytokine produced in the early response to sub-toxic concentrations of AgNPs, though IL-1 and IL-6 are induced later and at lower levels [11]. Additionally, in primary human peripheral blood monocytes, the release of mature IL-1β increased as particle size decreased [12]. AgNPs induced formation of the NLRP3 inflammasome and subsequent caspase-1 activation and cleavage of preformed IL-1β. AgNPs caused cathepsin leakage from lysosomes and efflux of intracellular K^+, which induced super oxide accumulation within mitochondrial membranes and led to inflammasome formation. Smaller AgNPs (5 nm) produced more hydrogen peroxide than larger AgNPs (28 nm). A recent study also demonstrated that AgNPs (15 nm) induced signatures of endoplasmic reticulum stress in THP-1 cells, including degradation of the ATF-6 sensor, pyroptosis induction, NLRP-3 inflammasome activation, increased caspase-1 activity, IL-1β secretion, and formation of the apoptosis-associated speck-like protein containing a CARD domain pyroptosome [13]. Three-day exposure of AgNPs (5.9 nm) to mouse bone marrow-derived macrophages resulted in reduced mitochondrial membrane potential, phagocytic capacity, and nitric oxide production upon treatment with lipopolysaccharide (LPS) [14].

With TiO_2 NPs (80 nm), very few changes were induced in the J774A.1 murine macrophage cell line [15]. However, ZnO NPs (100 nm) altered phagocytic capacity by increasing the release of IL-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8, and upregulating the expression of monocyte chemoattractant protein (MCP)-1 and cyclooxygenase-2 genes in THP-1 human monocytic cells [16]. Phagocytosis was decreased in RAW264 murine monocytic cells exposed to either gold NPs (AuNPs, 10 nm) or SiO_2 NPs (10 nm) [17]. SiO_2 NPs (10 nm) also caused changes in proliferation, cell cycle, and cell morphology, which demonstrate that bioaccumulation of AuNPs and SiO_2 NPs within the macrophages may impair signature functions including bactericidal activity and cytokine production [17].

A proteomic study in RAW264.7 murine monocytic cells revealed a rather weak response of the oxidative stress response pathway to treatment with ZnO NPs, but a strong response in the central metabolism and proteasomal protein degradation pathways, confirming that carbohydrate catabolism and proteasomal function are critical determinants of sensitivity to zinc, which also induces DNA damage. Conversely, glutathione levels and phagocytosis appear unaffected at moderately toxic concentrations of ZnO NPs [18]. A similar disturbance in carbohydrate metabolism has been shown in our study of AgNPs (5 nm) in HepG2 human hepatoma cells [19]. Treatment with 5 nm AgNPs decreased glucose consumption in HepG2 cells, which accompanied the reduced expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which was restored in the presence of an ROS scavenger. These metabolic changes may also occur in immune cells, thereby contributing to their dysfunction.

In contrast to the stimulatory effects of other NPs, AuNPs (50 nm) reduced the release of TNF-α and ROS production in macrophages treated with LPS. In mouse splenic lymphocytes, AuNPs significantly reduced the release of IL-17 and TNF-α triggered by LPS, suggesting that AuNPs may affect the balance of cytokines upon infection [20]. Another AuNP (36 nm) study showed an inhibition of matrix metalloproteinase (MMP)-8 and MMP-9 activity in RAW264 cells at a non-toxic concentration [21]. This study suggests that MMP inhibition could be attributed to the surface charge of polyvinylpyrrolidone (PVP), the outer coating of NPs. The negative charge of the surface coating of PVP binds to Zn^{++} ions in the active site of MMPs, thereby inhibiting enzymatic activity.

The levels of endotoxin were negative or low enough not to influence cellular activity in some studies [10, 11, 12, 17, 19], whereas the levels of endotoxin were not described in other studies. One study using AuNPs from two different products from same company (Nanocomposix, U.S.A.) indicated that the BioPure AuNPs are endotoxin-free, while the Econix AuNP are not guaranteed to be free of endotoxin [20].

Dendritic Cells

Recent studies investigating the effect of EMNs in dendritic cells were performed based on the role of dendritic cells as a vaccine platform. TiO_2 (20-80 nm) and SiO_2 (14 nm) NPs led to MHC-II, CD80, and CD86 upregulation in mouse bone marrow-derived dendritic cells. Furthermore, these particles activated the inflammasome, leading to secretion of IL-1β, suggesting that TiO_2 NPs activated dendritic cells [22]. Another study showed that human peripheral blood mononuclear cells (PBMCs) treated with TiO_2 NPs (21 nm) and ZnO NPs (10 nm) increased cell death and caspase activity, and down-regulated Fcγ RIII (CD16) expression on NK cells [23].

Submicron amorphous SiO_2 NPs were efficiently internalized by mouse bone marrow-derived macrophages via an actin cytoskeleton-dependent pathway, and induced caspase-1 [24]. Smaller (30 nm-1,000 nm) SiO_2 NPs induced lysosomal destabilization, cell death, and IL-1β secretion at markedly higher levels than did 3,000 nm-10,000 nm particles. Consistent with these *in vitro* results, intra-tracheal administration of 30 nm SiO_2 NPs in C57BL/6N mice caused more severe lung inflammation than 3,000 nm particles, as shown by higher production of pro-inflammatory cytokines and neutrophil infiltration in bronchoalveolar lavage (BAL) fluid [24]. Taken together, these results suggest that the size of SiO_2 NPs affects immune responses.

Surface modification of ENMs also influences their effects on dendritic cells. AuNPs (13-80 nm) with either

positive or negative surface charge were coated with polyethylene glycol (PEG), PVA, or a mixture of both to investigate uptake and cell response in human peripheral blood monocyte-derived dendritic cells [25]. Limited uptake was observed for PEG-COOH AuNPs, but a significant increase in TNF-α release was induced. In contrast, (PEG+PVA)-NH_2 and PVA-NH_2 AuNPs were internalized to a higher extent and caused IL-1β secretion, which suggests that surface modification influences internalization of NPs in dendritic cells.

The levels of endotoxin were negative [23, 25], whereas the presence of endotoxin was not assessed in other studies. In one study, NPs were baked at 220°C for 18 hours to destroy potential contaminating endotoxins, but endotoxin levels were not tested [22].

Mast Cells

Mast cells mediate allergic immune responses through degranulation of pre-formed mediators such as histamine. Mouse bone marrow-derived mast cells treated with AgNPs (20 nm and 110 nm) led to release of granule contents [26], suggesting either exacerbation of allergic disease or an antitoxin reaction to NPs. In another study, AgNPs induced degranulation in RBL-2H3 cells (rat basophil/mast cell line), which was confirmed by the increase of intracellular Ca^{++} and histamine [27].

In a study investigating the effect of ZnO (30 nm, 200 nm) and TiO_2 NPs (25 nm) on degranulation in RBL-2H3 cells and primary mouse bone marrow-derived mast cells, ZnO NPs markedly inhibited both histamine and β-hexosaminidase release, whereas TiO_2 NPs did not [28]. The inhibitory effects of ZnO NPs on mast cells were inversely proportional to particle size and dispersion status. More recently, ZnO NPs (200 nm) were found to inhibit the production of inflammatory cytokines such as IL-1β, IL-6, and TNF-α in HMC-1 cells (human mast cell line) treated with phorbol 12-myristate 13-acetate and A23187 [29]. In these cells, caspase-1 and NF-κB activation was abolished by treatment with ZnO NPs, and the expression of receptor interacting protein-2 and IκB kinaseβ (IKKβ) were reduced. Another study showed that exposure to TiO_2 NPs (60 nm) increased both histamine secretion and cytosolic Ca^{++} levels in RBL-2H3 cells. The increase in intracellular Ca^{++} levels resulted from an extracellular Ca^{++} influx via membrane L-type Ca^{++} channels, as well as oxidative stress induced by TiO_2 NPs [30].

The levels of endotoxin were not described in mast cell studies [26-30]. Therefore, the possibility that the contaminated endotoxin may trigger mast cell activation could not be excluded.

Endothelial Cells

In human umbilical vein endothelial cells (HUVECs), SiO_2 NPs (62 nm) induced ROS generation and caused oxidative damage followed by the production of malondialdehyde, the inhibition of super oxide dismutase and glutathione peroxidase, and a decrease in mitochondrial membrane potential [31]. The migration and adhesion of monocytes to endothelial cells are essential processes in early atherosclerogenesis. A study investigating the effect of ZnO NPs (20 nm) and TiO_2 NPs (21 nm) on HUVECs and THP-1 cells revealed that ZnO NPs induced the secretion of MCP-1, leading to the migration of THP-1 monocytes and adhesion to HUVECs [32]. Moreover, exposure to ZnO NPs upregulated the expression of membrane scavenger receptors of modified LDL and increased cholesterol uptake in THP-1 monocytes/macrophages. This study suggests that ZnO NPs, but not TiO_2 NPs, increase macrophage cholesterol uptake, which may enhance atherosclerogenesis and accelerate foam cell formation. However, another study in primary endothelial cell lines derived from human aorta and HUVECs showed that TiO_2 NPs (38 nm) increased adhesion molecule (ICAM-1, VCAM-1, E-selectin) expression and the release of MCP-1 and IL-8, indicating that TiO_2 NPs can induce endothelial inflammatory responses [33].

In another study, AgNPs (35 nm) were cytotoxic and rapidly stimulated ROS production in cultured human microvascular endothelial cells and endothelial colony-forming cells [34]. In HUVECs, exposure to AgNPs (65 nm) inhibited proliferation, damaged the cell membrane, and induced apoptosis. At the same time, the expression of inflammatory cytokines, adhesion molecules, and chemokines were increased in response to the ROS production. These results suggest that exposure to AgNPs may be a potential hazardous factor for early atherosclerotic development [35].

In HUVECs, AuNP (65 nm) induced the translocation of cytosolic Nrf2 to the nucleus, and its binding to the antioxidant-response element located in the E2 enhancer region of the hemeoxygenase (HO)-1 gene promoter. Knockdown of Nrf2 expression inhibited AuNP-induced HO-1 expression. AuNPs enhanced nuclear translocation of the Nrf2 protein, leading to Nrf2 binding to the HO-1 E2 enhancer promoter region to induce HO-1 gene expression. This study demonstrates that AuNPs may contribute to the anti-inflammatory response in human vascular inflammatory conditions [36].

Taken together, all of the above studies suggest that the balance between oxidative stress and anti-oxidant mechanisms govern the fate of various immune cells. Collectively, ROS production seems to be critical for cell damage and immune cell activation at low concentrations of ENMs. However, the levels of endotoxin were not described in endothelial cell studies [31-36]. Therefore, also in this case we cannot exclude the possibility that contaminating endotoxin may influence the cellular reactions to NPs.

EFFECT OF NPS ON THE SYSTEMIC IMMUNE SYSTEM

In addition to *in vitro* assays, *in vivo* studies by different administration routes are necessary for the proper evaluation of the immunological effects of NPs. NPs appear to induce a specific immunotoxic pattern consisting of induction of inflammation in normal animals and aggravation of pathologies in disease models.

TiO_2 NPs (21 nm) administration in Sprague-Dawley rats by intratracheal instillation for 4 weeks did not result in significant changes in the level of T_H1 (IL-2, INF-γ) or T_H2 (TNF-α, IL-6) cytokines [37]. However, another study showed that intragastric administration of TiO_2 NPs (5-6 nm)

in CD-1 mice for 90 days resulted in increased spleen and thymus size, splenocyte apoptosis, and increased levels of MIP-1α, MIP-2, eotaxin, monocyte chemotactic protein-1, IFN-γ, vascular cell adhesion molecule-1, IL-13, IFN-γ-inducible protein-10, migration inhibitory factor, and CD69 [38].

To determine the pulmonary toxicity of occupationally relevant ZnO NPs, Sprague-Dawley rats were exposed to ZnO NPs (50 nm) via intratracheal instillation. After 72 hours, ZnO NPs altered zinc balance and increased total cell numbers, neutrophils, the level of lactate dehydrogenase and total protein in BAL fluid, and the level of 8-hydroxy-2'-deoxyguanosine accompanying inflammatory pathology in the lungs. These findings highlight the occupational health effects for industrial workers exposed to ZnO NPs [39]. A single intraperitoneal administration of ZnO NPs (<50 nm) in combination with ovalbumin (OVA) increased the serum levels of OVA-specific IgG1 and IgE in BALB/c mice. In addition, levels of IL-2, IL-4, IL-6, and IL-17 were increased in splenocytes, while the levels of IL-10 and TNF-α were decreased. Macrophages and B cells showed higher expression of MHC class II molecules, whereas higher expression of CD11b was observed only in macrophages of mice treated with ZnO NPs or ZnO NPs/OVA. The lungs and spleen had increased eosinophils and mast cells, and myeloperoxidase activity in the lungs was increased [40].

A systemic toxicity study of AgNPs (20 nm and 100 nm) in Wistar derived WU rats via intravenous administration for 28 days showed a severe increase in spleen size and weight due to increased cell number. The most toxic effect was the suppression of NK cell activity in splenocytes, as well as decreased IFN-γ and IL-10 production by concanavalin-A stimulated splenocytes. IL-1β production was increased, whereas production of IL-6, IL-10, and TNF-α was decreased by lipopolysaccharide-stimulated spleen cells. Serum IgM, IgE, and blood neutrophilic granulocytes levels were increased [41].

An experiment investigating the effect of AuNPs (2-50 nm) in *ex vivo* studies using murine antibody-generating cells against haptoglobin (5B1B3 B cells, splenocytes fused with myeloma cells) indicated that treatment with AuNPs upregulated B lymphocyte-induced maturation protein 1 and down regulated paired box 5 to stimulate IgG secretion [42]. At the same study the immunization of BALB/c mice with peptide-conjugated AuNPs showed that AuNPs were capable of enhancing humoral immunity in a size-dependent manner.

As illustrated in the studies discussed above, the effect of NPs on various immune systems depends on size, surface modification, and most significantly, exposure route and duration. The effect of endotoxin contamination *in vivo* studies is not clarified because only one study has declared that NPs are free from endotoxin [40], whereas other studies did not describe the endotoxin levels.

ANIMAL MODELS OF ALLERGIC OR INFLAMMATORY DISEASES

Of particular interest in this review is the possibility of NPs to trigger overt reactions in individuals predisposed to allergic or inflammatory metabolic diseases.

The effect of SiO_2 NPs (55 nm) on preexisting allergic contact dermatitis (ACD) was investigated in an oxazolone-induced ACD model in SKH1 mice. Following administration of SiO_2 NPs for 5 days, no effects were observed in all clinical (transepidermal water loss and the erythema) and histologic (the degree of inflammation, epidermal thickness) examinations; serum levels of IgE were not significantly altered, suggesting that SiO_2 NPs did not affect the course of ACD [43]. However, intranasal instillation of SiO_2 NPs (90 nm) during OVA sensitization caused a dose-dependent enhancement of allergic airway manifestations in BALB/c mice [6]. In this study, OVA-specific serum IgE, airway eosinophil infiltration, mucous cell metaplasia, and T_H2 and T_H17 cytokine gene and protein expression were increased. These results suggest that airway exposure to SiO_2 NPs at lower doses in allergic mice could enhance allergen sensitization upon secondary allergen exposures [6]. Another study suggested opposing results. Treatment with OVA-loaded SiO_2 NPs modified the immune response with significantly lower serum levels of OVA-specific IgE and higher IgG levels in BALB/c mice, which is promising for the potential of SiO_2 NPs in therapeutic applications [44].

Repeated topical treatment of both ears and the back of NC/Nga mice, which are models for atopic dermatitis (AD), with a mixture of mite extract and SiO_2 NPs induced AD-like skin lesions. Measurements of ear thickness and histologic analyses revealed that cutaneous exposure to only SiO_2 NPs (20 nm) did not evoke AD-like lesions. Instead, concurrent cutaneous exposure to mite allergens and SiO_2 NPs resulted in the low-level production of allergen-specific IgG, including both the T_H2-type IgG1 and T_H1-type IgG2a subclasses (please note that this T_H1/T_H2 subclass specificity only refers to the mouse and is not present in man). Minimal changes in allergen-specific IgE concentrations were observed. These data suggest that SiO_2 NPs themselves do not directly affect the allergen-specific immune response after concurrent topical application of NPs and allergen. However, when present in allergen-adsorbed agglomerates, SiO_2 NPs led to a low IgG/IgE ratio, a key risk factor of human atopic allergies [45].

When administered in combination with OVA in BALB/c mice, TiO_2 NPs (28 nm) promoted a T_H2 dominant immune response with high levels of OVA-specific IgE and IgG1 in serum and an influx of eosinophils, neutrophils, and lymphocytes in BAL fluid. The TiO_2 NPs induced a significantly higher level of OVA-specific IgE than the standard adjuvant $AlOH_3$ [46]. Further, transdermal exposure to TiO_2 NPs of different sizes (15, 50, or 100 nm) aggravated AD-like skin lesions in NC/Nga mice [47]. The enhanced effect was associated with an overproduction of IL-4 in the skin and total IgE and histamine in serum, which was triggered by house dust mite allergens. TiO_2 NPs decreased the local expression of IFN-γ in the presence of allergen. No differences in size were observed following TiO_2 treatment. TiO_2 NPs alone also increased histamine levels in serum and IL-13 expression in the ear. A separate study determined the ability of TiO_2 NPs to modulate sensitization induced by dinitrochlorobenzene (DNCB) in BALB/c mice receiving subcutaneous injections of TiO_2 NPs (15-25 nm) before receiving DNCB. Injection of NPs resulted in cell proliferation in the lymph node, increased the level of IL-4, and decreased

IL-10 production in DNCB treated animals. This demonstrates that administration of TiO_2 NPs increases dermal sensitization to DNCB by augmenting the T_H2 response [48].

Nasal instillation of TiO_2 NPs (294 nm) for 9 months (chronic exposure) resulted in atherogenesis coupled with pulmonary inflammation in CD-1 (ICR) mice. The inflammation accampanying increased levels of serum triglycerides, total cholesterol, low-density lipoprotein cholesterol, ROS, NADPH oxidases 4, C-reaction protein, E-selectin, adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1), MCP-1, and plasminogen activator inhibitor-1, and reduced levels of serum high-density lipoprotein cholesterol, nitric oxide, and tissue plasminogen activator, suggested that long-term exposure to TiO_2 NPs is associated with atherosclerosis [49]. Another study in *Apoe*$^{-/-}$ mice showed that treatment with TiO_2 NPs (anatase, 5-10 nm) intratracheally for six weeks resulted in significant differences between the high dose group of TiO_2 NPs and control group. Levels of C reactive protein, nitric oxide, endothelial nitric oxide synthases, and total cholesterol were increased. The results also showed that the ratios of plaque area to luminal area and lipid-rich core area to plaque area in the medium and high dose groups were significantly increased, suggesting a potential role of TiO_2 NPsin the progression of atherosclerosis [50].

Genetic background is another critical factor to the immunological response to TiO_2 NPs (21 nm) inhalation. Two different inbred rat strains, the Dark Aguoti (DA), susceptible to chronic inflammatory disorders, and the Brown Norwegian (BN), susceptible to atopic allergic inflammation, were compared. Airway hyper-reactivity and production of inflammatory mediators of a T_H1 immune response were significantly increased only in DA rats. Sensitization of the rats induced a prominent OVA-specific-IgE and IgG response in the BN rat, while DA rats only showed an increased IgG response. The level of neutrophils and lymphocytes increased upon exposure to TiO_2 NPs in the airways of DA rats but were unchanged in BN rats. These results indicate that the genetic background plays a role in both immune response and airway reactivity to NPs [51].

The AD model using BALB/c mice demonstrated that ZnO NPs, but not micro-sized ZnO, are able to reach into the deep layers of the allergic skin. Although ZnO NPs diminish local skin inflammation in the mouse AD model, ZnO NPs induced systemic production of IgE antibodies, suggesting that topically applied ZnO NPs possess adjuvant activity [52]. A recent study evaluated the effect of inhalation of TiO_2 (30-50 nm), ZnO (21-25 nm),and SiO_2 (7-34 nm) NPs, and showed that the concentrations of total IgE, OVA-specific IgE, and OVA-specific IgG1 in serum increased in C57BL/6N mice treated with ZnO, but not TiO_2 or SiO_2 NPs. These results suggest that ZnO NPs have the potential to aggravate allergic reactions [53].

In another study assessing the allergenicity of AgNPs in a BALB/c mice model of asthma, a continuous exposure of AgNPs (33 nm) with OVA increased the enhanced pause, evoked neutrophil, lymphocyte, and eosinophil infiltration into the airways, and elevated the levels of IgE and leukotriene E4, IL-13, and oxidative stress (8-hydroxy-2'-deoxyguanosine) [54]. The protein profiles in BAL fluid and serum after inhalation of AgNPs (33 nm) in allergen provoked BALB/c mice revealed that metabolic, cellular, and immune system processes were associated with pulmonary exposure to AgNPs. Significant allergic responses were observed after exposure of AgNPs in control and allergic mice, as determined by OVA-specific IgE. These data suggest that inhaled AgNPs may regulate immune responses in the lungs of both control and allergic mice [55]. However, other studies have reported inhibitory effects of AgNPs on immune responses. For example, AgNPs (6 nm) ameliorated OVA-stimulated inflammatory cells, airway hyper-responsiveness, and levels of IL-4, IL-5, and IL-13 in C57BL/6 mice. In addition, NF-κB levels in lungs and ROS production in cells from BAL fluid were decreased by the administration of AgNPs [56]. These results indicate that AgNPs may attenuate antigen-induced airway inflammation and hyper-responsiveness. For endotoxin, only two papers declared that nanoparticles are endotoxin-free [44, 51], while others did not report endotoxin levels.

SUGGESTION OF ANIMAL MODELS TO ASSESS TOXICITY OF METAL OR METAL OXIDE NPS

We suggest the following animal models to investigate the precise immune-toxic or immune-regulatory functions of NPs.

Asthma

Acute allergen challenge model: BALB/c mice are the commonly used strain because they develop a good T_H2 immune response [57], although other strains (C57BL/6 and A/J) have also been used successfully [58]. Although OVA is a frequently used allergen, house dust mite and cockroach extracts can also be used [59].

Chronic allergen challenge model: Chronic allergen challenge models involve repeated exposure of the airways to relatively low levels of allergen for up to 12 weeks; co-administration of an adjuvant is not always required. OVA [60, 61], as well as environmentally relevant allergens such as house dust mite extract or grass pollen [59, 62], are also used.

Atopic Dermatitis

AD models induced by epicutaneous sensitization: In BALB/c or C57BL/6 mice, the repeated epicutaneous sensitization of tape-stripped skin with OVA is performed [63]. Also, BALB/c mice exposed to epicutaneous application of the recombinant mite allergen Der p8 show dermatitis symptoms with epidermal hyperplasia and spongiosis, skin infiltration with $CD4^+$ and $CD8^+$ cells, and a skewed T_H2 response both locally and systemically [64], which are similar to the effects of epicutaneous sensitization with OVA.

Hapten-induced mouse models of AD: Multiple challenges with oxazolone or trinitrochlorobenzene to the skin of hairless mice over an extended period cause the skin inflammation to shift from a typical T_H1 immune response to a chronic T_H2 response that is similar to human AD [65, 66].

Genetically engineered mouse models of AD: IL-4 transgenic mice over expressing IL-4 in the skin develop spontaneous pruritus and chronic dermatitis at the age of 4

months [67]. Conditional thymic stromal lymphopoietin (TSLP) transgenic mice may be also used [68, 69].

Spontaneous mouse models of AD: The NC/Nga mice are another model mimicking human disease in which skin symptoms develop spontaneously in response to exposure to various environmental allergens [70].

Atherosclerotic Mice

Although there are few animal studies investigating metal oxide/metal ENMs and atherosclerosis at the present, we recommend that this animal study be included for the assessment of immune-toxicity of ENMs. An *Apoe*$^{-/-}$ mice develop spontaneous plaques when fed a normal chow diet, and exhibit accelerated plaque formation when fed a western diet chow [71]. *Ldlr* $^{-/-}$ mice exhibit a lipoprotein profile similar to human disease, and may be used with or without a cholesterol and fat rich diet [72].

CONCLUSION

To assess the effects of NPs in the immune system, highly specialized experimental animal models should be adopted. In this article, we suggest prototypic animal models of atopic dermatitis, asthma, and atherosclerosis. Sub-toxic exposure levels, exposure routes, genetic background, and predisposition to immunological disorders should be carefully considered in order to precisely mimic real-life exposure scenarios and environmental factors.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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